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Bile Salts and Glycine as Cogerminants for *Clostridium difficile* Spores[▽]

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Spore formation by *Clostridium difficile* is a significant obstacle to overcoming hospital-acquired *C. difficile*-associated disease. Spores are resistant to heat, radiation, chemicals, and antibiotics, making a contaminated environment difficult to clean. To cause disease, however, spores must germinate and grow out as vegetative cells. The germination of *C. difficile* spores has not been examined in detail. In an effort to understand the germination of *C. difficile* spores, we characterized the response of *C. difficile* spores to bile. We found that cholate derivatives and the amino acid glycine act as cogerminants. Deoxycholate, a metabolite of cholate produced by the normal intestinal flora, also induced germination of *C. difficile* spores but prevented the growth of vegetative *C. difficile*. A model of resistance to *C. difficile* colonization mediated by the normal bacterial flora is proposed.

Clostridium difficile, a gram-positive spore-forming bacterium, is the leading cause of antibiotic-associated diarrhea, pseudomembranous colitis, and toxic megacolon (12). Perturbation of the colonic flora, a side effect of treatment with broad-spectrum antibiotics, potentiates sensitivity to *C. difficile* infection (18). The normal flora is thought to protect against *C. difficile* by occupying potential sites of colonization (35, 45). Once established in the intestinal tract, *C. difficile* elicits disease upon the secretion of two toxins, TcdA and TcdB (38). Due to the prevalence of *C. difficile*-associated disease in hospitals and relapse in patients treated for *C. difficile* infection, it has been estimated that the total financial burden on the health care system for the treatment of *C. difficile*-associated disease in the United States is approximately $3.2 billion per year (24).

Vegetative cells of *C. difficile* are exquisitely sensitive to oxygen. To survive outside the anaerobic environment of the large bowel, the bacterium has to be in the spore form. Thus, it is generally accepted that the spore form of *C. difficile*, acquired from the environment, initiates disease. Since toxins are produced by cells, not spores, the spores presumably germinate in the gastrointestinal tract, grow out as vegetative cells, and produce toxin. Any *C. difficile* bacteria that are excreted by the host, however, have to be in the spore form to survive for long periods (15). Although the morphological changes during sporulation are very similar in *Clostridium* and *Bacillus* species, sporulation and germination in *Clostridium* species are not as well studied as those in the model organism *Bacillus subtilis*. In brief, sporulation is initiated under conditions of nutrient limitation and leads to formation of an asymmetrically placed division septum that divides the cell into two unequal compartments, each of which contains one copy of the chromosome. The larger, mother cell compartment then engulfs the forespore and helps the forespore mature (11). The addition of a peptidoglycan cortex and several layers of coat proteins precedes release into the environment by lysis of the mother cell (9).

Once released from the mother cell, the spore is metabolically dormant but highly resistant to many types of environmental insult. When conditions become suitable for growth, the spores germinate and grow out as vegetative cells. In *B. subtilis*, germination can be induced by L-alanine or by a mixture of asparagine, glucose, fructose, and potassium ions. Receptors involved in sensing these environmental cues are GerA, GerB, and GerK (14, 21). After the germinant is sensed, a large depot of calcium dipicolinate (Ca^{2+}-DPA) is released, the core hydrates, the cortex is degraded, and metabolism begins (34). Homologs of GerA, GerB, and GerK exist in several *Bacillus* species as well as in many *Clostridium* species but are absent in *C. difficile*, suggesting that *C. difficile* responds to different kinds of environmental cues (26, 33). In fact, spore germination in different species is induced by a variety of germinants. For instance, for *Bacillus megaterium* spores, L-proline is a germinant (31), while purine ribonucleosides and amino acids act as cogerminants for *Bacillus anthracis* spores (13). Germination and outgrowth of *C. difficile* spores have not been studied in depth, due in part to the absence of genetic tools. Specifically, the germination step, classically defined as the change in the optical density caused by spore rehydration and Ca^{2+}-DPA release, has not been studied as an independent phenomenon. Previous work showed that taurocholate, a bile salt, enhances colony formation by *C. difficile* spores recovered from environmental surfaces and stool (2, 40, 44). Similarly, treatment of *C. difficile* spores with lysozyme and thioglycolate enhances colony formation (16, 43). These effects on colony formation are clear, but it is difficult to discern what specific effects the treatments might have on germination.

Bile is produced by the liver and stored in the gall bladder. To aid in digestion, the gall bladder secretes bile into the duodenum, where it helps to absorb fat and cholesterol. The primary bile produced by the liver consists mainly of cholate and chenodeoxycholate conjugated with either taurine or glycine (Fig. 1) (30). During passage through the distal ileum, bile is actively reabsorbed and recycled to the liver. However, 400

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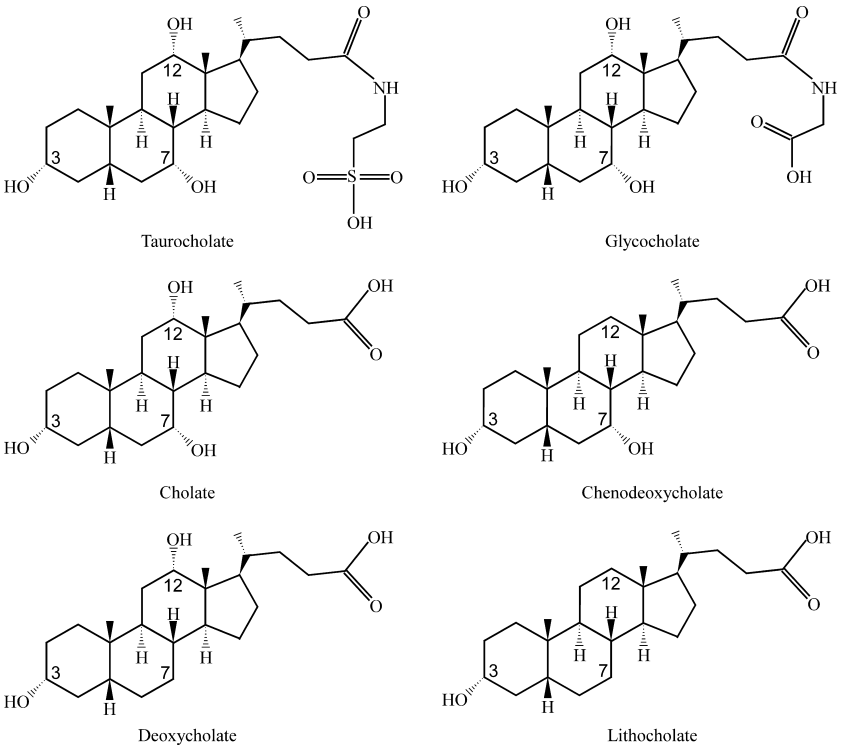

FIG. 1. Structures of common primary and secondary bile acids. The primary bile salts cholate and chenodeoxycholate typically are conjugated with taurine or glycine (only taurocholate and glycocholate are shown). The normal intestinal microbial flora deconjugates the tauryl and glycyl group from cholate and chenodeoxycholate. The deconjugated primary bile salts are further metabolized by the microbial flora to deoxycholate and lithocholate, respectively.

to 800 mg of bile passes daily from the ileum into the cecum, where it becomes a substrate for biotransforming reactions by the normal, benign bacterial flora (36, 37). Many different species of bacteria, including *Clostridium perfringens* (6), express on their cell surfaces bile salt hydrolases (BSHs), which remove the conjugated amino acid from the primary bile salt. This hydrolysis reaction appears to proceed to completion, inasmuch as conjugated primary bile salts are essentially undetectable in the human cecum (7, 23). Though some *Clostridium* species express BSHs, none have been described for *C. difficile*, and no open reading frame product with homology to BSHs in other species is present.

Unconjugated primary bile salts are taken up by a small percentage of bacterial species in the colon (30). One of these species, *Clostridium scindens*, actively transports unconjugated, primary bile salts into the cytosol and, through a series of enzymatic reactions, converts cholate and chenodeoxycholate to the secondary bile salts deoxycholate and lithocholate, respectively (Fig. 1) (20, 41, 42). These secondary bile salts are secreted from the bacteria into the extracellular environment and are eventually excreted by the host.

We report here that *C. difficile* spores germinate rapidly in vitro when exposed to taurocholate but only in a rich medium that does not by itself induce efficient germination. Using a defined medium, we found that glycine and taurocholate act as cogerminants and are sufficient to induce germination of *C. difficile* spores. Secondary bile salts induced germination but also inhibited outgrowth of the germinated spores. These results form the basis for a new model to explain the role of the normal flora in preventing *C. difficile* infection.

MATERIALS AND METHODS

Strains and growth conditions. *C. difficile* CD196 and *C. perfringens* SM101 were described previously (27, 46). *C. difficile* UK14 was obtained from Dale Gerding's laboratory and was isolated during the epidemic *C. difficile* outbreak at Stoke-Mandeville Hospital, Aylesbury, Buckinghamshire, England (Meridian Biosciences strain number SM8-6865). All strains were grown in BHIS (brain heart infusion [Difco] supplemented with yeast extract [5 mg/ml] and L-cysteine [0.1%]) at 37°C under anaerobic conditions in a Coy Laboratory anaerobic chamber.

Preparation of *C. difficile* spores. Sporulation of *C. difficile* was induced on BHIS agar as described previously (8). Briefly, an overnight *C. difficile* culture in BHIS medium was diluted in fresh medium to an optical density at 600 nm (OD_{600}) of 0.2. A 150-μl portion of this suspension was spread on 5 ml BHIS agar in each well of a six-well tissue culture dish. The culture was incubated anaerobically for 4 to 7 days. To determine spore colony formation, samples from the plates containing mixed populations of spores and vegetative cells were resuspended in BHIS and heated to 60°C for 20 min to kill vegetative cells before cooling, diluting, and plating on BHIS medium. For use in germination assays, spores were purified by a method similar to that used by Akoachere and colleagues (1). The vegetative-cell–spore mixture was collected by flooding each well of the six-well dish with ice-cold sterile water. After five washes with ice-cold water, the bacteria were suspended in 20% (wt/vol) HistoDenz (Sigma, St. Louis, MO). This suspension was layered onto a 50% (wt/vol) HistoDenz solution in a centrifuge tube, and the tube was centrifuged at 15,000 × *g* for 15 min to separate spores from vegetative cells. The purified spores, collected at the bottom of the centrifuge tube, were washed twice with ice-cold water to remove traces of HistoDenz and resuspended in water.

In vitro response of *C. difficile* spores to bile salts. To determine the response time of *C. difficile* spores to taurocholate, spores were produced as described

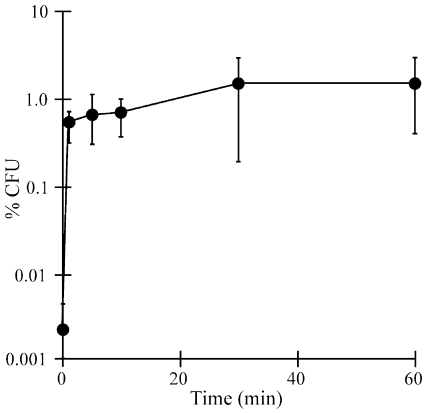

FIG. 2. Rate of response of *C. difficile* spores to taurocholate. *C. difficile* spores were suspended in water containing 0.1% taurocholate. At 1, 5, 10, 30, and 60 min, spores were serially diluted and plated on BHIS agar in the absence of taurocholate. Colonies were enumerated after overnight growth, and data were compared to those for spores spread on BHIS(TA). Data are means from three independent experiments, and error bars represent 1 standard deviation from the mean.

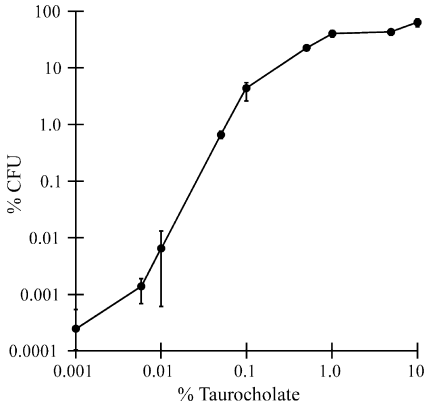

FIG. 3. Amount of taurocholate required for efficient recovery of *C. difficile* spores. *C. difficile* spores were incubated in water containing increasing concentrations of taurocholate, serially diluted, and plated on BHIS agar in the absence of taurocholate. Colonies were enumerated after overnight growth, and data were compared to those for spores spread on BHIS(TA). Data points are means from three independent experiments, and error bars represent 1 standard deviation from the mean.

above. Vegetative bacteria were heat killed by incubation for 20 min at 60°C. The heat-treated spores were washed three times in water to remove traces of nutrients and returned to the anaerobic chamber to allow subsequent colony formation. Spores were resuspended in water, and either taurocholate, glycocholate, cholate, deoxycholate, ursodeoxycholate, or chenodeoxycholate (Sigma, St. Louis, MO) was added to 0.1%. At various times, samples were removed, serially diluted, and plated on BHIS agar. One sample was removed prior to the addition of taurocholate and spread on BHIS and BHIS(TA) (BHIS plus 0.1% taurocholate). The latter platings served as the negative and positive controls for colony formation, respectively. After overnight growth, colonies were enumerated (CFU), and the number was compared to that obtained on BHIS(TA).

To determine the amount of taurocholate needed to induce colony formation by *C. difficile* spores, spores were produced, heated, and washed as described above. Heat-treated *C. difficile* spores were resuspended in water containing various concentrations of taurocholate. After a 10-minute incubation, the suspensions were serially diluted in BHIS and plated on BHIS agar. After overnight growth, colonies were enumerated, and the number was compared to that obtained by overnight growth on BHIS(TA).

Germination of *C. difficile* spores. Germination of *C. difficile* spores was measured by diluting purified *C. difficile* spores to an OD_{600} of 0.3 to 0.4 in BHIS alone or BHIS containing 1% bile salts (taurocholate, glycocholate, cholate, or deoxycholate). For experiments in complete defined medium, a mixture of salts [0.3 mM $(NH_4)_2SO_4$, 6.6 mM KH_2PO_4, 15 mM NaCl, 59.5 mM $NaHCO_3$, and 35.2 mM Na_2HPO_4] was used to buffer the spores and putative germinants (17). The OD_{600} was determined immediately (time zero) and at various time points during incubation at room temperature. The ratios of the optical densities at the various time points to the optical density at time zero were plotted against time.

Statistical analysis. All assays listed above were performed in triplicate, and data are reported as means and standard deviations from three independent experiments.

RESULTS

Time of in vitro taurocholate exposure required to enhance colony formation by *C. difficile* spores. There have been reports over the past 25 years that the bile salt taurocholate enhances the recovery of *C. difficile* spores from environmental surfaces and stool (2, 28, 44). We confirmed that inclusion of 0.1% taurocholate in BHIS agar plates enhances the recovery of *C. difficile* spores approximately 10^5-fold. To know how long an exposure to taurocholate is required to increase colony formation, spores and vegetative bacteria were heated at 60°C for 20 min and washed three times with water to remove traces of nutrients that may affect germination. Spores were then returned to the anaerobic chamber and treated with 0.1% taurocholate in water (Fig. 2). At the indicated times, samples were removed, diluted in BHI medium, and plated on BHIS agar (without taurocholate). One sample was not incubated in vitro with taurocholate but was plated directly on BHIS(TA). This sample served as a reference for 100% recovery. As shown in Fig. 2, as little as 1 minute of exposure to taurocholate resulted in an increase in spore recovery from 0.0025% to about 1%. Further incubation did not significantly enhance colony formation by *C. difficile* spores. These results demonstrate that *C. difficile* spores respond very rapidly to taurocholate, suggesting that taurocholate may be a germinant.

Effects of taurocholate concentration on spore colony formation. Because colony-forming ability in response to 0.1% taurocholate did not reach that of spores plated on BHIS(TA) directly, we tested the in vitro recovery of spores in response to different concentrations of taurocholate. Spores were prepared as described above, washed with water to remove traces of nutrients, incubated for 10 min with taurocholate at concentrations ranging from 0.001% to 10%, and plated on BHIS agar without taurocholate. Spore colony-forming ability was compared to that of spores plated directly on BHIS(TA). Incubation of spores with 0.001% taurocholate resulted in colony formation by approximately 0.0002% of the total number of spores (Fig. 3). This efficiency of colony formation was routinely seen in the absence of any taurocholate and can vary approximately 10-fold (Fig. 2). Increasing the concentration of taurocholate increased the plating efficiency of *C. difficile* spores (Fig. 3). Incubation for 10 min in 10% taurocholate resulted in a plating efficiency that was 60% of that seen with continuous exposure to 0.1% taurocholate (Fig. 3). This result suggests that continuous exposure to a low concentration of taurocholate significantly enhances colony formation even further or that the effect of taurocholate is enhanced when spores are in contact with a solid surface or both.

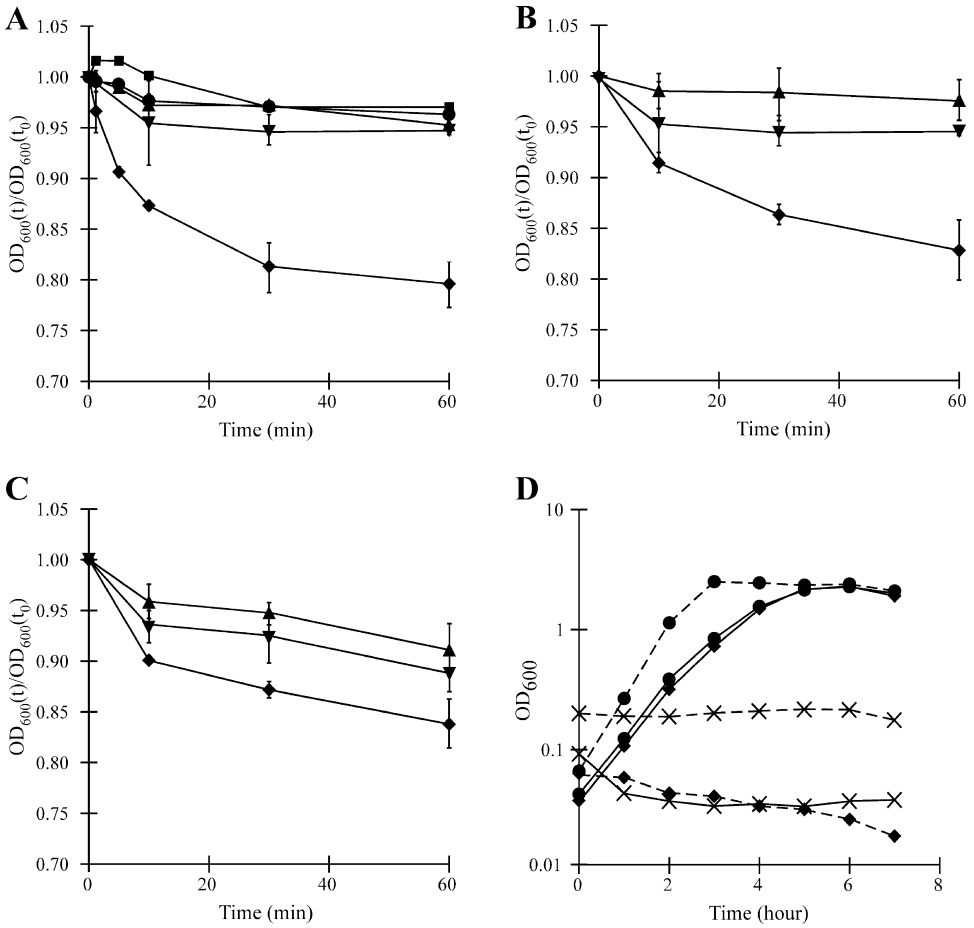

FIG. 4. Effect of primary bile salts on the germination and growth of *C. difficile*. (A) *C. difficile* spores were purified and suspended in BHIS alone (●), in 1% taurocholate in buffer (▼), or in BHIS containing 1% taurocholate (◆), 1% glycocholate (▲), or 1% cholate (■). The ratios of the optical densities at various time points to the starting optical density are plotted against time. (B) *C. difficile* CD196 spores were purified and suspended in buffered glycine (1.3 mM) (▲), buffered 1% taurocholate (▼), or buffered glycine plus taurocholate (◆). Germination was measured as for panel A. (C) *C. difficile* UK14 spores were purified and suspended in buffered glycine (1.3 mM) (▲), buffered 1% taurocholate (▼), or buffered glycine plus taurocholate (◆). Germination was measured as for panel A. (D) Vegetative *C. difficile* (solid lines) and vegetative *C. perfringens* (dashed lines) were grown in BHIS alone (●), BHIS(TA) (◆), or BHIS plus 0.1% chenodeoxycholate (×). Error bars represent 1 standard deviation from the mean.

Germination in response to primary bile salts. In *B. subtilis* and other species, the addition of a germinant to spores results in a change from phase bright (refractile) spores to phase dark spores due to the release of Ca^{2+}-DPA and rehydration of the spore (22). This transition is the first stage of germination, can be measured as a decrease in the optical density of the culture, and is typically used to define germinants. To see if taurocholate enhances colony formation by increasing the rate or extent of spore germination, *C. difficile* spores were incubated in phosphate buffer (pH 7.2) with 1% taurocholate or in buffer alone. One percent taurocholate was chosen because this concentration enabled colony formation by about 30% of the total number of spores during in vitro exposure (Fig. 3). At regular intervals, the OD_{600} was measured and plotted against time. By this measure, taurocholate did not induce germination of *C. difficile* spores (Fig. 4A). To see if spores germinate in BHIS medium but become arrested before acquiring the ability to form colonies, spores were suspended in BHIS medium, and the optical density of the culture was monitored. No significant decrease in optical density was seen (Fig. 4A), indicating that spores do not germinate in BHIS alone. This result is in agreement with earlier observations that *C. difficile* spores do not efficiently form colonies in standard media without additional reagents (44). The addition of 1% taurocholate to BHIS resulted in a rapid decrease in the optical density to about 85% of the starting value, with a continued decrease to about 80% of the starting value (Fig. 4A). This is similar to what is seen for germination of *Clostridium botulinum* spores; the rate of germination appears to be higher in *C. difficile* (3). These results suggest that taurocholate and an unknown component of BHIS medium are cogerminants of *C. difficile* spores; neither cogerminant activates germination by itself.

Spore germination and colony formation in response to primary bile salts. As taurocholate is a primary bile salt produced by the liver and secreted to aid in digestion, we hypothesized that other primary bile salts might also induce germination of *C. difficile* spores. To test the effect of other primary bile salts on colony formation, spores were plated on BHIS agar

TABLE 1. Cholate derivatives induce colony formation by *C. difficile* spores

Cholate derivative added to BHIS[a]	CFU recovery (%)[b]	
	Mean	SD
TA	100	
GA	86.3	17.2
CA	75.1	10.3
CDCA	<0.0001[c]	
UA	<0.0001[c]	

[a] Spores were serially diluted and spread on BHIS agar containing 0.1% taurocholate (TA), glycocholate (GA), cholate (CA), chenodeoxycholate (CDCA), or ursodeoxycholate (UA).
[b] Data are percentages relative to the value obtained with BHIS(TA) and are means of three independent experiments.
[c] CFU for CDCA and UA were below the limit of detection for this experiment.

TABLE 2. Colony formation of *C. difficile* spores

Treatment[a]	CFU recovery (%)[b]	
	Mean	SD
None	100	
TA	1.27	0.38
CDCA	0.0012	0.001
DCA	1.48	0.27

[a] Spores were treated in vitro with 0.1% taurocholate (TA), chenodeoxycholate (CDCA), or deoxycholate (DCA), serially diluted, and spread on BHIS agar.
[b] Data are percentages relative to the value obtained with BHIS(TA) and are means of three independent experiments.

containing 0.1% cholate, glycocholate, chenodeoxycholate, ursodeoxycholate, or taurocholate. Interestingly, only cholate derivatives (cholate, glycocholate, and taurocholate) stimulated efficient colony formation by *C. difficile* spores (Table 1). Chenodeoxycholate and ursodeoxycholate, the 7β epimer of chenodeoxycholate, were not effective in this assay (Table 1). The primary bile salts cholate and glycocholate were then compared to taurocholate for germination-inducing ability. Incubation of *C. difficile* spores in BHIS with glycocholate or cholate did not result in any significant decrease in the optical density of the culture even when the assay was carried out to 6 h (Fig. 4A and data not shown). Thus, glycocholate and cholate enhance colony formation by spores on plates but do not stimulate germination per se by the assay used.

Glycine is a cogerminant for *C. difficile* spores. To identify the component of BHIS that induces germination with taurocholate, the medium was divided into BHI and yeast extract. In the presence of taurocholate, both BHI and yeast extract induced germination of *C. difficile* spores (data not shown). We used the defined medium described by Karlsson and colleagues (17) to identify the specific compound or compounds that induce germination. When spores were suspended in complete defined medium with taurocholate, the optical density of the culture decreased to the same extent as in BHIS(TA) (data not shown). When this medium was divided into it constituents and subconstituents, we found that spores suspended in buffer containing glycine germinated in the presence of taurocholate but not in its absence (Fig. 4B). These results indicate that glycine and taurocholate are cogerminants.

To test whether glycine and taurocholate act as cogerminants for another strain of *C. difficile*, we tested a strain, UK14, isolated during an outbreak at Stoke-Mandeville Hospital in the United Kingdom. When *C. difficile* UK14 spores were suspended in buffer containing glycine or taurocholate alone, a small decrease in the optical density of the culture was observed (Fig. 4C). When both glycine and taurocholate were present, the efficiency of germination was enhanced (Fig. 4C). These results confirmed our initial results in *C. difficile* CD196 that taurocholate and glycine act as cogerminants for *C. difficile* spores.

Effect of primary bile salts on the growth of *C. difficile*. We next tested whether vegetative cells of *C. difficile* are able to grow in the presence of the primary bile salts. As expected, *C. difficile* was able to grow in BHIS containing 0.1% taurocholate, glycocholate, or cholate to the same extent as in BHIS medium alone (Fig. 4D and data not shown). *C. difficile* was unable to grow in the presence of chenodeoxycholate. Therefore, the absence of growth demonstrated by the data in Table 1 can be explained by growth inhibition by chenodeoxycholate. In contrast to *C. difficile*, *C. perfringens* SM101 was unable to grow in the presence of either 0.1% taurocholate or 0.1% chenodeoxycholate (Fig. 4D) (10). *C. perfringens* SM101 was able to grow to wild-type levels in the presence of glycocholate and cholate (data not shown). These results suggest that *C. difficile* may have evolved a mechanism to resist the toxic effects of taurocholate in addition to sensing taurocholate as a germinant.

Chenodeoxycholate inhibited the growth of vegetative cells of *C. difficile* and *C. perfringens*. To test whether transient exposure to chenodeoxycholate induces colony formation by *C. difficile* spores, spores were suspended in water containing 0.1% chenodeoxycholate for 10 min, serially diluted in BHIS medium, and plated on BHIS agar in the absence of chenodeoxycholate. Exposure to chenodeoxycholate did not induce colony formation by *C. difficile* spores (Table 2). These results suggest that *C. difficile* spores only germinate in BHIS and form colonies in response to cholate derivatives (taurocholate, glycocholate, and cholate).

Deoxycholate induces colony formation but prevents the growth of *C. difficile*. In the cecum, the primary bile salts cholate and chenodeoxycholate are metabolized by the normal bacterial flora to the secondary bile salts deoxycholate and lithocholate, respectively (Fig. 1) (30, 36). During the passage of *C. difficile* through the intestinal tract, spores and vegetative cells undoubtedly come into contact with secondary bile salts. We tested whether deoxycholate can induce the germination or recovery of *C. difficile* spores. Lithocholate could not be tested, as it is insoluble in water. When *C. difficile* spores were incubated in vitro with 0.1% deoxycholate, serially diluted, and plated on BHIS agar, colony-forming ability was indistinguishable from that of spores incubated with taurocholate (Table 2). These results suggest that deoxycholate, like other cholate derivatives, induces colony formation by *C. difficile* spores (Tables 1 and 2). Incubation of *C. difficile* spores in BHIS with 1% deoxycholate resulted in a small drop in OD_{600} that after 60 min was not significantly more than the change in OD of spores in BHIS alone (Fig. 5A).

C. difficile does not grow in the presence of deoxycholate

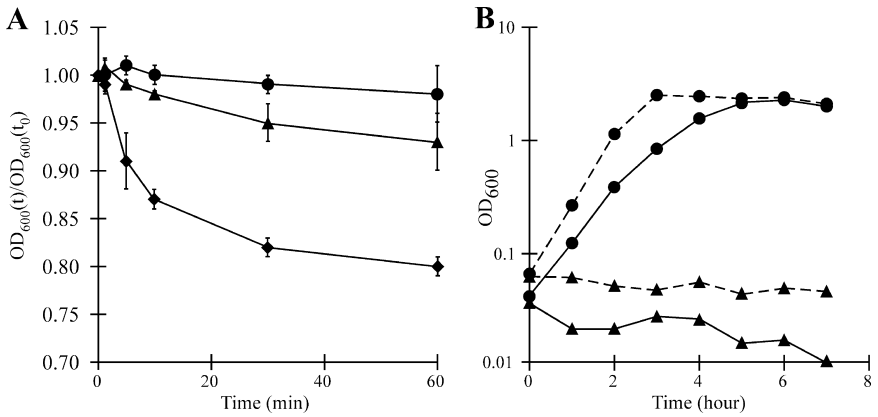

FIG. 5. Effect of deoxycholate on the germination and growth of *C. difficile*. (A) *C. difficile* spores were purified and suspended in BHIS alone (●) or in BHIS(TA) (◆) or 1% deoxycholate (▲). (B) Vegetative *C. difficile* (solid lines) and vegetative *C. perfringens* (dashed lines) were grown in BHIS alone (●) or BHIS plus 0.1% deoxycholate (▲). Error bars represent 1 standard deviation from the mean.

(43). To quantify this effect, we measured the growth of *C. difficile* and *C. perfringens* in BHIS containing deoxycholate. Although *C. difficile* grew well in medium containing taurocholate, neither *C. difficile* nor *C. perfringens* grew in the presence of deoxycholate (Fig. 5B).

DISCUSSION

The initiation of germination is characterized by a change from a phase bright spore to a phase dark spore (39), and this criterion is often used for defining germinants. Interaction of a germinant with its germination receptor triggers a series of enzymatic reactions that leads to the release of Ca^{2+}-DPA, core rehydration, cortex degradation, and the eventual outgrowth of a vegetative cell (34). The absence in *C. difficile* of germinant receptors homologous to those in *B. subtilis* raises the possibility that *C. difficile* spores respond to unique germinants. We have shown that *C. difficile* spores germinate rapidly in rich medium in the presence of taurocholate. When germination is measured spectrophotometrically, only *C. difficile* spores that are incubated in rich medium containing taurocholate transition from phase bright spores to phase dark spores, a hallmark of germination (Fig. 4A). The ability to germinate and form colonies on plates requires only a brief exposure to taurocholate, but continuous exposure during colony formation is more effective than transient exposure (Fig. 2). When *C. difficile* spores are plated on BHIS agar containing cholate or glycocholate, they form colonies with the same efficiency as spores plated on BHIS agar containing taurocholate (Table 1). Further, when spores are incubated briefly with the secondary bile salt deoxycholate, a compound that inhibits the growth of *C. difficile*, spores are subsequently able to form colonies on BHIS agar in the absence of any germinant. All of these results indicate that cholate conjugates and metabolites are cogerminants for *C. difficile* spores. However, they must activate germination at different rates, because only taurocholate was capable of inducing rapid germination (loss of refractility) while glycocholate, cholate, and deoxycholate induced colony formation with the same efficiency as taurocholate. Northfield and McColl have shown that postprandial concentrations of bile acids along the ileum range from 10 mM (0.41% to 0.52%) in the upper ileum to 2 mM (0.082% to 0.1%) in the lower (23). Therefore, the concentrations of bile that stimulate colony formation that are used in this study are likely to be physiologically relevant.

If exposure to either glycocholate, cholate, or deoxycholate in liquid medium stimulates subsequent colony formation by *C. difficile* spores on agar medium, why do these compounds not induce germination as detected spectrophotometrically? When *C. difficile* spores are treated in vitro with 0.1% taurocholate, only about 1% of the total spores present germinate and resume vegetative growth when serially diluted and plated on agar medium without taurocholate (Fig. 2 and 3). However, the same spore preparation plated on agar medium containing 0.1% taurocholate induces the germination and growth of approximately 100-fold more spores. This suggests either that spores must encounter both cogerminants simultaneously or that the germination of *C. difficile* spores is enhanced by contact with a solid support. The latter hypothesis is interesting because germination in the host likely involves a semisolid support in the cecal environment. The spore morphology of *C. difficile* CD196 is not known. However, an exosporial layer is present on the surface of spores of *C. difficile* strain ATCC 43594 (25). Upon the initiation of germination, the exosporium of *C. difficile* spores produces small filaments that attach to the solid medium and prevent the spores from being washed away (25). This type of surface attachment may facilitate the contact between taurocholate, the nutrient-rich medium, and the spore, thus enhancing germination in the laboratory environment.

Chenodeoxycholate, a primary bile salt, did not induce colony formation by *C. difficile* spores (Table 2). As chenodeoxycholate has limited solubility in water, it is possible that not enough was dissolved to induce germination. An alternative hypothesis is that the 12α-hydroxyl group of cholate derivatives is an important side chain that *C. difficile* spores recognize; chenodeoxycholate derivatives lack the hydroxyl group at the 12α position (Fig. 1 and Table 2). If this hypothesis is correct, the 12α position could be exploited to find potential inhibitors of *C. difficile* spore germination.

Neither glycine nor taurocholate has been previously de-

scribed as a germinant in the strict sense for spores of *Bacillus* or *Clostridium* species. The only other known approach to inducing germination of *C. difficile* in the laboratory is to treat spores with high concentrations of a reducing agent and lysozyme (16). This method of germination is not likely to be biologically relevant.

In bile, glycine is conjugated to cholate (Fig. 1). When glycocholate passes into the lower bowel, the glycyl group is deconjugated from cholate by the normal bacterial flora (5). The products of deconjugation are cholate and glycine. When deconjugated by the normal flora, the production of both cholate and glycine from glycocholate results in two compounds that are sufficient to stimulate germination and outgrowth of *C. difficile* spores.

The results presented here lead to a possible adjunct to the current model for colonization by *C. difficile* and the protective effect of the normal flora. When a normal, healthy host ingests *C. difficile* spores, the spores survive the passage through the stomach and pass through the duodenum and into the jejunum, where the concentrations of primary bile salts and nutrients are high (29, 30). *C. difficile* spores germinate in response to cholate derivatives and glycine. The germinated spores then pass through the ileum and finally into the anaerobic environment of the cecum. Here, certain members of the normal microbial flora metabolize the cholate derivatives that escape enterohepatic circulation to deoxycholate, a predominant bile salt in the feces of healthy humans (4, 19, 36). The deoxycholate produced prevents vegetative growth of *C. difficile*, and the host remains uncolonized. Spores that did not germinate during passage through the upper part of the digestive system may germinate in response to deoxycholate but are then unable to grow in the toxic environment. Upon antibiotic treatment, the normal microbial flora is perturbed and the species that are capable of 7α-dehydroxylation of primary bile salts are significantly reduced (32). This reduction would lead to an increase in the concentration of primary bile salts (cholate derivatives) and a decrease in the concentration of secondary bile salts (deoxycholate) in the cecum (32). This decrease in secondary bile acids may provide an environment in which *C. difficile* can grow and colonize. Moreover, the abnormal presence of primary bile acids in the large bowel may contribute to the extent of *C. difficile* colonization by providing a nontoxic germinant for spores that remain within the colon. Therefore, an important protective role the normal microbial flora plays may be that of metabolizing cholate derivatives to deoxycholate, an inhibitor of *C. difficile* growth. Standard therapy for *C. difficile* infection is treatment with vancomycin or metronidazole. It is not surprising that patients suffer relapses after completing treatment regimens with these drugs, since the microbial flora needs time to repopulate the colon and restore the normal balance of primary and secondary bile acids. We are currently testing this hypothesis and are aware of the possibility that chenodeoxycholate, a primary bile salt that inhibits growth, may act to protect against *C. difficile* colonization. If this model withstands further scrutiny, it would have significant therapeutic potential. For example, patients undergoing antibiotic therapy may benefit from supplementing their normal diets with deoxycholate or with probiotics containing bacterial species that are capable of removing the 7α-hydroxyl group from cholate.

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